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13. ABSTRACT ***(Maximum 200 Words)***

BAG-1 is a novel regulator of Hsp70 family molecular chaperones. The activity of many steroid hormone receptors, including the estrogen receptor, ERα, is known to be regulated by Hsp70 and Hsp90 family proteins. A carboxyl terminal domain of BAG-1 binds to the ATPase domain of Hsc70/Hsp70 and regulates its chaperone function. We have cloned two additional novel BAG-1 homologs (BAG-2, BAG-3) using yeast two hybrid screening with the Hsc70 ATPase doimain as a bait. In addition we have identified two more possible BAG homologs in the EST database. The fundamental hypothesis we propose to test is that BAG family proteins are important regulators of ER function in breast cancers. In the first year of this research proposal, we cloned full length cDNA of BAG family proteins. Predicted amino acid sequence shows that all member of these protein has the similar sequence in the c-terminal homologous domain and diverse sequence in N-terminus domain. Several kinds of antibody against BAG family proteins have been generated using GST fusion protein and we have preliminary data about the western analysis of breast cancer cell lines.

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FOREWORD

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PI - Signature 6/29/00 Date

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INTRODUCTION

Breast cancer is a hormonally driven disease. The estrogen receptor (ER) controls the response of the normal breast and breast cancers to estrogens. BAG1 is a novel regulator of estrogen receptor. During my investigation about BAG1, we found additional 4 human genes, which have c-terminal homology domain of BAG1. BAG family proteins are binding to Hsp70 molecular chaperone. And our data shows that deletion of Hsp70 binding domain of BAG1 lose it's ability to regulate ER. To understand the mechanism of ER regulation by BAG1, we proposed to examine how BAG family protein regulate breast cancer cell growth and ER activation. We cloned all full length cDNA of BAG family proteins and made constructs for biological and biochemical assay. Recently, we generated several antibodies against BAG family protein and tried to detect expression level in breast cancer cell lines. Our preliminary data suggested that BAG3 express very strongly in breast cancer cell lines. I believe studies of these proteins and their interactions with estrogen receptors will improve our understanding of the mechanisms that cause breast cancer and lay the foundation for devising novel strategies for improving therapy.

BODY

Task1. Deduce the Complete Primary Aminoacid Sequences of BAG-3, BAG-4 and BAG- 5. (Months 1-6)

a. Identify tissues or cell lines rich in mRNAs for BAG-2, BAG-3, BAG-4, and BAG-5 by Northern blotting.

b. Obtain full-length BAG-family cDNAs by conventional lambda phage library screening using ^{32}P-labeled hybridization probes or 5'RACE.

c. Determine DNA sequences of cDNAs and deduce complete ORFs of BAG-family proteins.

a. Confirm ORFs by in vitro translation experiments.

Completion of Task 1 has been reported as a genbank submission described below (Appendices). We used two kinds of libraries for cloning these cDNAs. One is Jurkat lambda ZAPII library and the other is HUCL (Stratagene, La Jolla). Detailed procedure has been reported in the 6 month report.

Task 2. Determine the biochemical effects of BAG-family proteins on Hsc70 chaperone function (Months 6-12).

a. Subclone cDNAs into pGEX4T-1 for production of GST fusion proteins.

PCR based deletion mutant has been made for these constructions using pGEX4T-1 vector. 5' primers has EcoRI (BamHI for BAG4) and 3' primer has XhoI site.

b. Produce and purify BAG-family proteins from bacteria.

Most of these fusion proteins are soluble, except BAG5BD2, BAG5BD4. We are trying to determine the condition of expression and purification of these proteins.

c. Perform biochemical assays of BAG-family protein effects on Hsc70 chaperone activity (refolding assays), ATPase activity, and ADP-ATP exchange.

Task 3. Map the domains within BAG-family proteins which are needed for interactions with Hsc70 (Months 13-16).

a. Create deletion mutant of BAG-1 family proteins by PCR mutagenesis of cDNAs.

See Task 2a. We already made several deletion mutants.

b. Subclone into pGEX and yeast two hybrid plasmids.

Same fragment from Task2a were used for yeast two hybrid vectors, pGilda and pJG4-5

c. Perform Hsc70 interaction assays.

Task 4. Examine effects of BAG-family proteins on Estrogen Receptor Function. (Months 17-20)

a. Subclone epitope tagged BAG-family cDNAs into pcDNA3 for expression in mammalian cells.

pcDNA3 myc vector was used for subcloning the fragments from Task2a. All construct have been made

b. Perform transient transfection reporter gene assays for ER function using estrogens and antiestrogens.

Task 5. Explore the effects of BAG-family proteins on in vitro behaviors of breast cancer cells (Months 21-36)

a. Choose cell lines for studies based on RNA blot analysis of endogenous levels of BAG-1, BAG-2, BAG-3, BAG-4, and BAG-5 expression.

b. Perform transfections and select for cell lines stably expressing BAG-1 family proteins.

c. Verify expression by immunoblotting, using epitope tags.

d. Examine effects of BAG-family protein over-expression on (a) steroids; (b) chemotherapeutic drugs; and (c) cell motility.

Task 6. Determine the incidence of BAG-family protein expression in breast cancers (months 12-36).

a. Immunize rabbits with recombinant BAG-family proteins or KLH,OVA-conjugated peptides and produce antisera (Months 0-24)

b. Characterize antibodies by immunoblotting and immunoprecipitation assays: Verify specificity and lack of cross-reactivity with other BAG-family proteins.

We made several antisera against recombinant BAG family proteins and two of those have been characterized. Bur 101 is anti BAG2 antibody and Bur 102 is anti BAG3 anti sera. Using these antibodies, we performed western blot analysis using breast cancer cell lines. Fig 3. 293 T cell was transfected by pcDNA3 myc BAG3 and BAG2. Anti myc monoclonal antibody (Santa Cruise Biotec), Bur 101 and Bur102 antibody were used to compare signals. Anti BAG2 and Anti BAG3 antibodies work on the western blot. Fig4 shows overexpression of BAG3 protein (~80kDa) in Breast cancer cell lines. Comparing to BAG3 expression, BAG2 protein (~25kDa) is pretty low level in breast cancer cell lines. Further characterization of these antibody will be necessary.

c. Analyze BAG-family mRNA and protein levels in breast cancer cell lines by RNA and immunoblotting (Months 25-30).

d. Analyze BAG-family gene expression in primary archival breast tumor specimens by in situ hybridization or immunohistochemistry (Months 30-36).

KEY RESEARCH ACCOMPLISHMENTS

1, Using partial cDNA as probes, we cloned full length cDNA of BAG3, BAG4, BAG5.
2, cDNAs have been amplified and subcloned into pGEX, pGilda, pJG4-5, pcDNA3 etc.
3, Purification of GST fusion protein of wild type and deletion mutants of BAG family proteins is undergoing.
4, Rabbit antisera have been generated and characterized.
5, Preliminary western blot was performed using breast cancer cell lines.

REPORTABLE OUTCOME

As listed in Appendices, GENBANK submission has been made by the nucleotide and peptide sequence data of BAG3, BAG4 and BAG5. GenBank accession number is listed in Appnedices.

CONCLUSIONS

As we spent most of the first year for cloning and determination of nucleotide sequence, we don't have many important results and implications about breast cancer biology. Determination of full length cDNA sequence and predicted amino acid sequence have been finished. Predicted amino acid sequence revealed similar sequence at the carboxyl-terminal region of each protein. But the N-terminal region has different peptide sequence.

BAG2 protein is not expressed very well in breast cancer cell lines by western blot. This protein might not very important in breast cancer cell biology. We always observe higher molecular weight band by BAG2 antibody. More characterization of this antibody and protein expression will be necessary about BAG2 project.

BAG3 protein has WW domain at the N-terminal region and proline rich sequence is located in the middle portion of this protein (fig.1). The WW domain has been found in many proteins, including Nedd4 ubiquitin ligase, oncoprotein YAP and so on. Although, physiological role of WW domain is not clear yet, recently many reports show the importance of WW domain as a binding domain of proline rich sequence(1). In the proline rich domain of BAG3, there are several PXXP motifs, which are reported as SH3 recognition sequences. From these feature of peptide sequence, we speculated that BAG 3 binds to partner proteins at the N-terminus and regulate the function of Hsp70 molecular chaperone at the C-terminus. It is interesting to find any cancer or growth related protein interaction with N-terminal region of BAG3(2). Our preliminary western blot shows overexpression of BAG3 protein in breast cancer cell lines (fig.4). We observed several extra band in BAG3 western blot. Shorter band might be the degraded protein and more careful characterization will be necessary. Basically BAG3 protein is overexpressed in most of the breast cancer cell lines. Interestingly, adreamycine resistant MCF-7 has less expression of BAG3 than parental cell. We are preparing RNA for northerblot analysis. Comparison between drug resistant cell lines and parental cell line might have some clue of the mechanism of upregulated expression of BAG3 in breast cancer cell lines.

The investigation of associated protein of BAG3 might be useful to understand the function of BAG3 protein. Recently, bcl-2 binding protein, bis, has been published and we found this protein is identical to BAG3. Their report showed BAG3/bis works

synergistically in preventing cell death with Bcl-2. It is interesting for us to examine Bcl-2 related breast cancer cell biology with BAG3.

BAG4 protein is also proline rich protein. There are no clear motif in this protein. After we cloned this protein as a homologue of BAG3 from EST data base (3), a gene encoding silence of death domain (SODD) was identified (4). SODD associated with TNFR1 and DR3 to prevent spontaneous death signaling. An amino acid sequence is identical to BAG4. At present, we are unable to show their published data about SODD by using our BAG4 cDNA clone. As we are using different system from this group, we should be more careful about this story. Recently SODD/BAG4 was reported as a overexpressed protein in pancreatic cancer (5). It is suggested that carcinogenesis inducing overexpression of BAG4/SODD in this type of cancer. The investigation of the mechanism of the upregulation of BAG4 in cancer cell might show an interesting result in not only pancreatic cancer but also breast cancer.

As I showed in fig.1, BAG5 has 4 possible BAG domain. We made several deletion mutants to see which BAG domain is important for the binding to 70kDa molecular chaperones (fig2). We are trying to purify all of these recombinant GST fusion proteins.

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APPENDICES

Nucleotide and Amino acid sequence

1. BAG3 nucleotide sequence and predicted amino acid sequence.

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Primates; Catarrhini; Hominidae; Homo. REFERENCE 1 (bases 1 to 2534)
AUTHORS Takayama,S., Xie,Z. and Reed,J.C. TITLE       An evolutionarily
conserved family of Hsp70/Hsc70 molecular
chaperone regulators
JOURNAL J. Biol. Chem. 274 (2), 781-786 (1999) MEDLINE 99091615
REFERENCE 2 (bases 1 to 2534)
AUTHORS Takayama,S. and Reed,J.C.
TITLE  BAG-family regulators of Hsp70/Hsc70
JOURNAL Unpublished
REFERENCE 3 (bases 1 to 2534)
AUTHORS Takayama,S. and Reed,J.C.
TITLE  Direct Submission
JOURNAL Submitted (28-SEP-1998) The Burnham, 10901 N. Torrey Pines Rd., La
Jolla, CA 92037, USA
REFERENCE 4 (bases 1 to 2534)
AUTHORS Takayama,S. and Reed,J.C.
TITLE  Direct Submission
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2. BAG4 nucleotide and predicted amino acid sequence

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ACCESSION AF095195
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KEYWORDS .
SOURCE human.
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Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo. REFERENCE 1 (bases 1 to 4285)
AUTHORS Takayama,S., Xie,Z. and Reed,J.C. TITLE An evolutionarily conserved family of Hsp70/Hsc70 molecular chaperone regulators
JOURNAL J. Biol. Chem. 274 (2), 781-786 (1999) MEDLINE 99091615
REFERENCE 2 (bases 1 to 4285)
AUTHORS Takayama,S. and Reed,J.C.
TITLE BAG-family regulators of Hsp70/Hsc70
JOURNAL Unpublished
REFERENCE 3 (bases 1245 to 1957)
AUTHORS Takayama,S. and Reed,J.C.
TITLE Direct Submission
JOURNAL Submitted (28-SEP-1998) The Burnham Institute, 10901 N. Torrey Pines Rd., La Jolla, CA 92037, USA
REFERENCE 4 (bases 1 to 4285)
AUTHORS Takayama,S. and Reed,J.C.
TITLE Direct Submission
JOURNAL Submitted (10-SEP-1999) The Burnham Institute, 10901 N. Torrey Pines Rd., La Jolla, CA 92037, USA
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Fig. 1 Structure of BAG family proteins

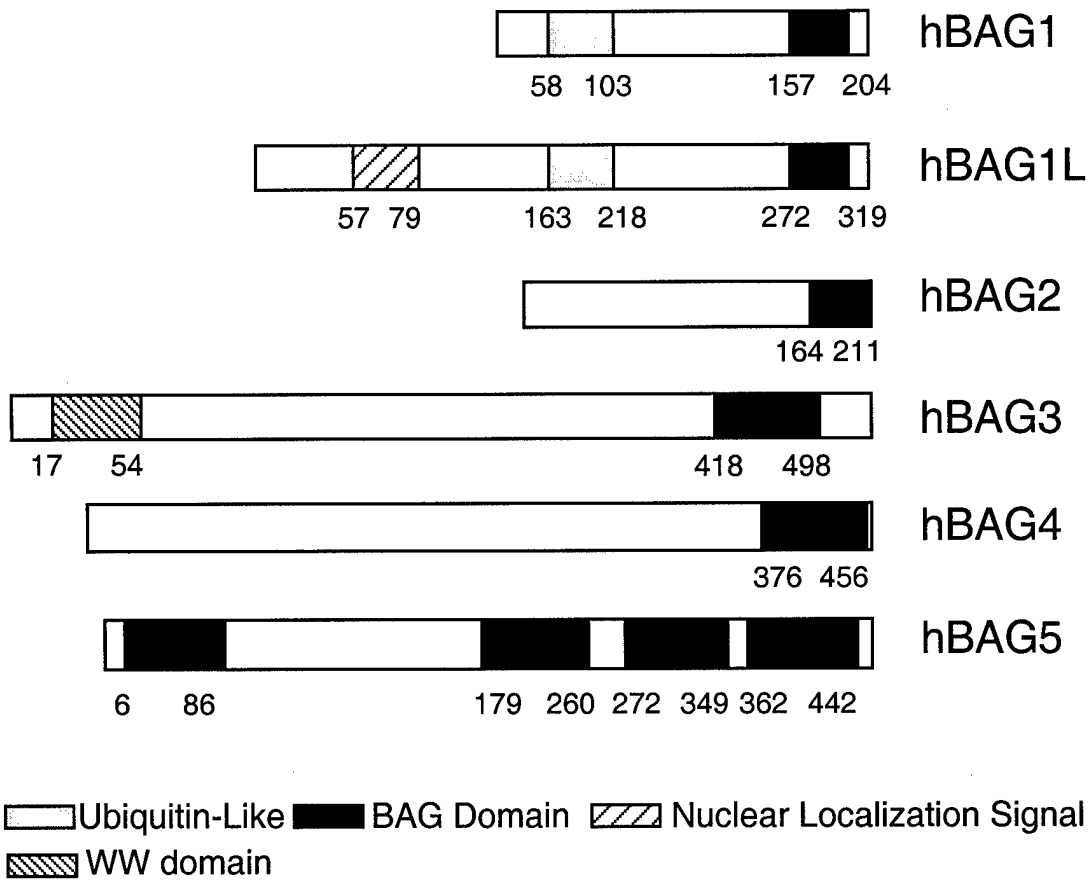
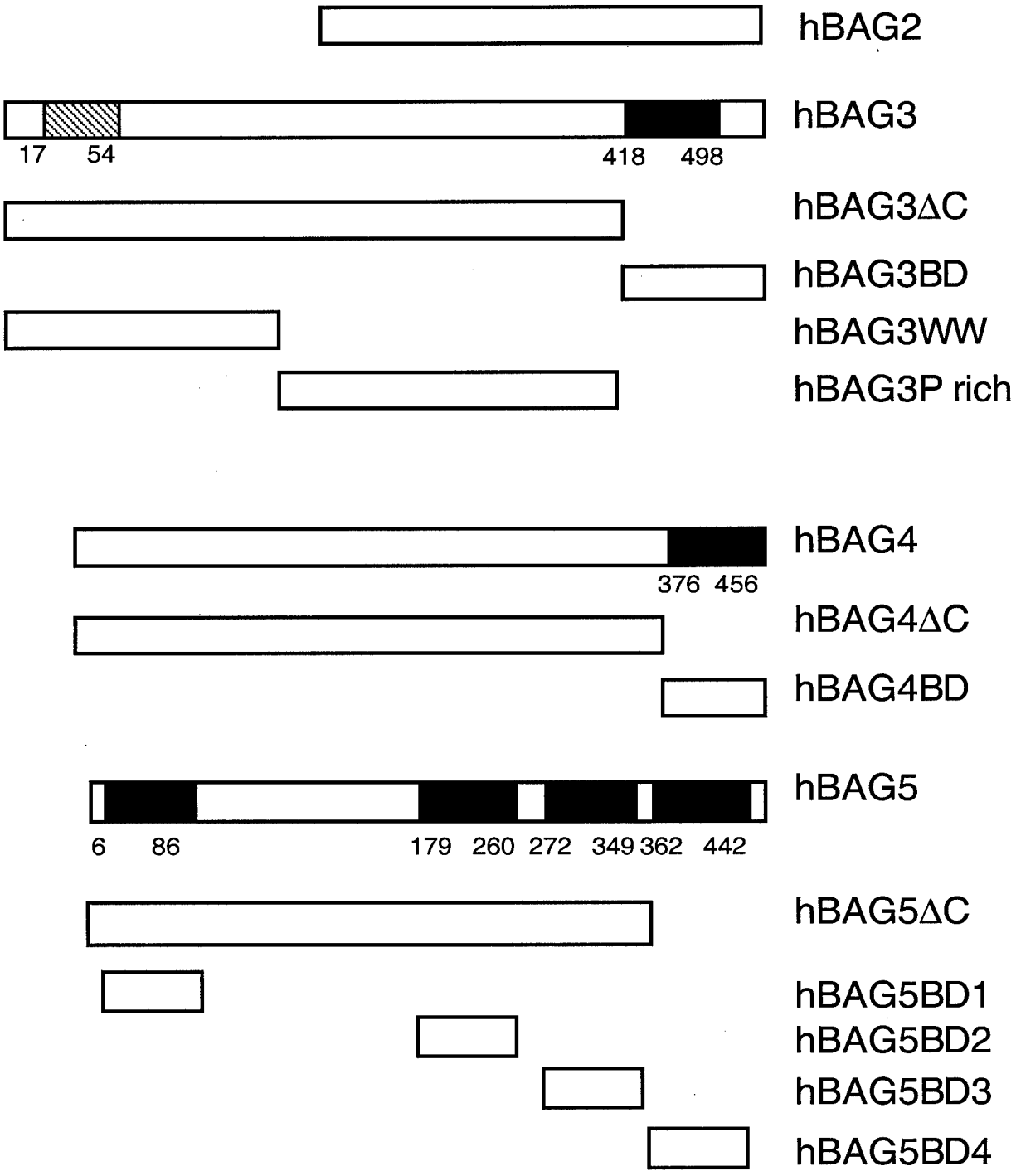


Fig. 2 PCR based deletion mutants.



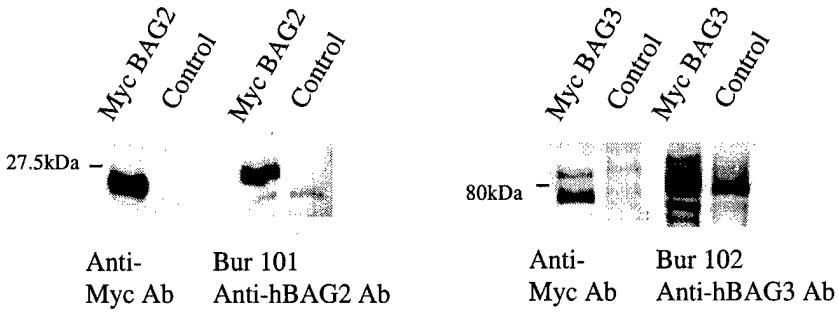


Fig.3 Characterization of anti BAG2 Bur101And anti BAG3 Bur102 antisera

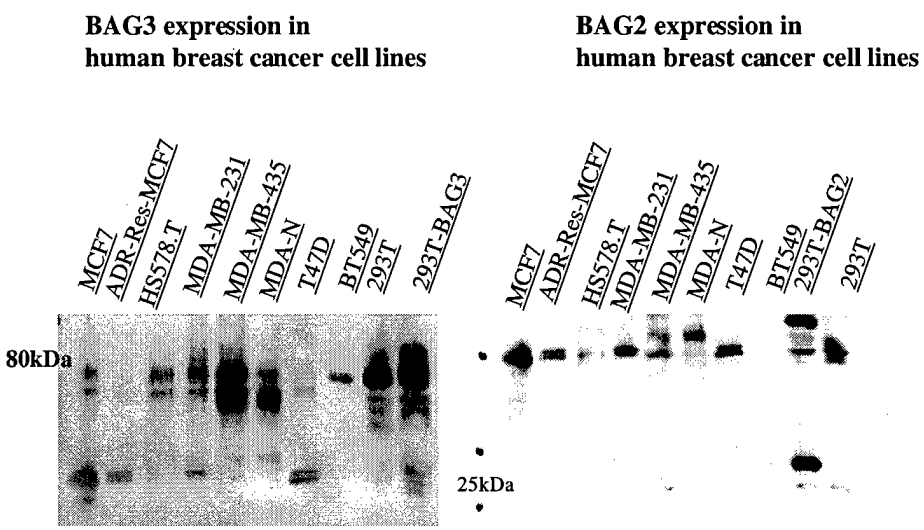


Fig.4 Western blot analysis of Breast cancer cell lines by anti BAG2 and BAG3 antibody